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Research paper

Inhibition of Na^+,K^+-ATPase by cisplatin and its recovery by 2-mercaptoethanol in human squamous cell carcinoma cells

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Na^+,K^+-ATPase (EC 3.6.1.37) is assumed to be involved in the transport of cisplatin [*cis*-diamminedichloroplatinum(II)] into cells and to act as a modulator of 5-fluorouracil (5-FU) in combination therapy of cisplatin and 5-FU. Whereas inhibition of Na^+,K^+-ATPase activity by cisplatin is expected to have effects on both anti-cancer therapy and nephrotoxicity, the inhibition mechanism remains to be elucidated. We studied the inhibition of Na^+,K^+-ATPase activity by cisplatin using an enzyme partially purified from Ca9-22 cells derived from a human squamous cell carcinoma of the gingiva. Cisplatin inhibited the Na^+,K^+-dependent ATP hydrolysis activity, and this inhibition depended on both the concentration of cisplatin and the preincubation time with cisplatin. The time-dependent inhibition was thought to be caused by a slow change of cisplatin from the inactive to the active form. We further tested the effect of cisplatin on the partial reactions of the enzyme, Na^+-dependent ATP hydrolysis and K^+-dependent *p*-nitrophenylphosphate hydrolysis activities to determine which step in the reaction sequence of Na^+,K^+-ATPase was inhibited. Cisplatin inhibited both activities depending on its concentration and the preincubation time, whereas the Na^+-dependent ATP hydrolysis activity was inhibited even at lower concentrations. Formation of a phosphointermediate of Na^+,K^+-ATPase was also inhibited by cisplatin depending on the concentration and preincubation time. Cisplatin (500 μM) and 8-fold higher concentration of 2-mercaptoethanol (2-ME; 4 mM) prevented inactivation of the enzyme by cisplatin, and the Na^+,K^+-ATPase activity inhibited by pretreatment with cisplatin was also recovered almost completely by 2-ME. These results suggest that the active form of cisplatin inhibits the Na^+,K^+-ATPase activity by inhibiting the formation of a phosphointermediate of the enzyme and that the inhibition by cisplatin is arrested by an addition of thiol group. [© 1999 Lippincott Williams & Wilkins.]

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Introduction

Cisplatin is one of the most important and effective anti-cancer agents used in the treatment of ovarian, stomach, lung, testicular, and head and neck carcinomas.[1] It is assumed that cisplatin acts in two ways. One is to enter the nuclei and to form cross-links with DNA in cancer cells, threreby causing a direct inhibition of growth of cancer cells.[1–4] The other is to work as a modulator of 5-fluorourcil (5-FU) in combination therapy of cisplatin and 5-FU.[5,6] The hypothesis is as follows: cisplatin inhibits Na^+,K^+-ATPase activity[7] which transports methionine into cells, thereby lowering the methionine concentration in the cells.[8,9] The activation of methionine synthesis simultaneously elevates reduced folate level and this elevation activates thymidylate synthase (TS). Finally an active metabolite of 5-FU binds to TS covalently, inhibits it and thereby causes inhibition of DNA synthesis. Some reports argue against this hypothesis,[10,11] and the details of the anti-cancer mechanism of cisplatin and its drug accumulation remain unclear.

Many studies have dealt with the relationship between cisplatin and Na^+,K^+-ATPase from the standpoint of drug accumulation[12–16] and prevention of nephrotoxicity.[17–20] Na^+,K^+-ATPase is a membrane enzyme which translocates sodium and potassium ions across the cell membrane, utilizing hydrolysis energy of ATP as the driving force. The sodium gradient formed by the transport is used for translocating glucose, amino acids and other nutrients into cells. Therefore, it is easy to understand that methionine transport is mediated by Na^+,K^+-ATPase and that

transport of cisplatin into the cells may also be mediated by this enzyme. Physiologically Na^+,K^+-ATPase present in the kidney is known to regulate fluid reabsorption and electrocyte movement by establishing an ionic gradient across epithelial membranes;[21,22] therefore, nephrotoxicity caused by cisplatin administration is possibly due to inhibition of Na^+,K^+-ATPase activity.

Several reports have already suggested that transport of cisplatin into cells is mediated by Na^+,K^+-ATPase[12–16] and that Na^+,K^+-ATPase activity is inhibited by cisplatin.[8,5,7,17–20] These findings reveal that the relationship between cisplatin and Na^+,K^+-ATPase is complicated, since cisplatin disturbs the source of its own translocation energy. It is thus important to elucidate the inhibition mechanism of Na^+,K^+-ATPase activity by cisplatin, since this inhibition may cause the following effects: (i) a decrease in the accumulation of cisplatin in cancer cells and a reduction in its direct anti-cancer effect, (ii) inhibition of methionine transport into cancer cells and modulation of the anti-cancer effect of 5-FU; and in particular, (iii) inhibition of kidney Na^+,K^+-ATPase activity causing nephrotoxicity. Recently the reaction mechanism of Na^+,K^+-ATPase has been elucidated.[23,24] According to the standard Albers–Post reaction sequence, binding of cytoplasmic ATP to the KE_2 (potassium-bound enzyme) form induces the E_1 (molecular species of Na^+,K^+-ATPase which favors Na^+ and ATP) conformation that favors the binding of sodium ions from the cytoplasm; binding of cytoplasmic magnesium ion catalyzes the transfer of the terminal phosphate of ATP bound to the active site of the enzyme and forms a phosphoenzyme intermediate. The phosphoenzyme releases sodium ions into the extracellular medium and then accepts extracellular potassium ions. Binding of potassium ions, therefore, accelerates dephosphorylation of the enzyme and it returns to its initial state, KE_2. The purpose of our study was to analyze the inhibition of Na^+,K^+-ATPase activity by cisplatin with a focus on the reaction mechanism of the enzyme. For this purpose we used the partially purified Na^+,K^+-ATPase from a human carcinoma cell line.

Materials and methods

Cell line and culture conditions

Ca9-22 cells, derived from a human squamous cell carcinoma of the gingiva, were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (Life Technologies, Grand Island, NY) and 60 μg/ml kanamycin sulfate at 37°C with a 95% air/5% CO_2 atmosphere; the cells were subcultured approximately every 4 days by trypsin-EDTA treatment.

Drugs and chemicals

Cisplatin was obtained from Sigma (St Louis, MO), DMEM from ICN Pharmaceuticals (Costa Mesa, CA), sodium dodecyl sulfate (SDS) from BDH (Poole, UK). Ouabain was obtained from Wako Pure (Osaka, Japan). All other drugs were obtained from Sigma.

Enzyme preparation

Na^+,K^+-ATPase from Ca9-22 cells was prepared by treating the microsomal fraction of the cells with SDS and by separating them with step glycerol gradient basically as described by Jørgensen[25] with a modification.[26] Between 4 and 5 days after confluence, the cells were washed twice with solution A (250 mM sucrose containing 1 mM Tris–EDTA, pH 7.4) and collected by scraping the dishes with a rubber policeman into solution A. Then, the cells were ultrasonicated and centrifuged at 5500 *g* for 40 min at 4°C. The supernatant was transferred into fresh tubes and centrifuged at 35 000 *g* for 50 min at 4°C for separation of the microsomal fraction as a pellet. The pellet was homogenized with a Teflon homogenizer and suspended in solution A at a concentration of 2 mg/ml. SDS treatment solution was prepared to give final concentrations of 0.25 mg/ml SDS, 3 mM ATP-2Na, 7 mM Tris, 12.5 mM imidazol, 1.3 mM cysteine-HCl (pH 7.6) and 0.5 mM H_4EDTA, when the same volume of the solution as that of the microsomes was added. The microsome fraction was incubated with SDS under continuous stirring for 45 min at room temperature. After incubation the mixture was layered onto 44% (w/w) glycerol with 25 mM imidazole, 1 mM H_4EDTA and 2.6 mM cysteine-HCl (pH 7.6), and then centrifuged at 25 000 *g* for 16 h at 5°C. After centrifugation, a pellet and a turbid band were obtained. The pellet was homogenized, and suspended in a small volume of storage medium containing 24 mM imidazol, 0.5 mM H_4EDTA, 16 mM HEPES, 2 mM dithiothreitol and 3.258 mM glycerol at pH 7.4.[26] The turbid band in the supernatant was diluted with a solution containing 25 mM

imidazole, 1 mM H_4EDTA and 2.6 mM cysteine-HCl (pH 7.6), and recentrifuged at 86 500 *g* for 60 min at 5°C. The pellet was homogenized and suspended as described above. We tested the ratio of SDS to protein concentration of microsome in SDS treatment and found that 0.25 mg SDS to 1 mg protein gave the best preparation. In our best preparation the recovery of Na^+- and K^+-dependent ATP hydrolysis activity in the microsomal fraction was about 84%, and both fractions of Na^+,K^+-ATPase activities were more than 70% ouabain sensitive. Specific activities of the enzyme were 1.2–1.5 μmol of P_i/mg protein/min.

Measurement of Na^+,K^+-ATPase activity

To determine the optimal reaction condition for Na^+,K^+-ATPase activity of Ca9-22 cells, we examined the concentrations of ligands sufficient to saturate the ouabain-sensitive ATPase activity. The examination was performed by changing one ligand concentration while concentrations of other ligands were fixed at high levels sufficient to saturate the activity. The activity was saturated at the concentrations of 40 mM NaCl, 8 mM KCl, 6 mM $MgCl_2$ and 5 mM ATP, respectively (data not shown). The optimum pH for the activity was 7.2–7.5. The activity was inhibited completely by 1 mM ouabain, a specific inhibitor of Na^+,K^+-ATPase activity (data not shown). From these results the concentrations of ligands, and the pH value in the reaction mixture and the ouabain concentration in the blank experiments were determined. In fact, concentrations of the various components in the 0.3 ml reaction mixture were 1–5 μg of the enzyme, 25 mM sucrose, 0.1 mM EDTA, 100 mM Tris-HCl at pH 7.5, 40 mM NaCl, 8 mM KCl, 6 mM $MgCl_2$ and 5 mM ATP with or without 1 mM ouabain. The reaction was started by the addition of ATP and the reaction mixture was incubated for 20–30 min at 37°C; then the reaction was stopped by the addition of 0.3 ml of 12% SDS. The released inorganic phosphate was detected by the method of Chifflet *et al.*[27] Briefly, 0.6 ml of the solution containing 3% ascorbic acid, 0.5 N HCl and 0.5% ammonium molybdate was added to the 0.6 ml reaction mixture with SDS, which was left for 3–10 min at room temperature. Then, 0.9 ml of a solution containing 2% sodium citrate, 2% sodium metaarsenite and 2% acetic acid was added to the mixture, which was then incubated for 10 min at 37°C. The developed color was read at 850 nm spectrophotometrically.

Measurement of Na^+-ATPase activity

Na^+-dependent ATP hydrolysis activity, partial reaction of Na^+,K^+-ATPase, was measured. The various components in the 0.3 ml reaction mixture were 10–12 μg of enzyme, 25 mM sucrose, 0.1 mM EDTA, 100 mM Tris-HCl at pH 7.5, 1 mM NaCl, 3 mM $MgCl_2$ and 2 mM ATP with or without 1 mM ouabain. The reaction was started by the addition of ATP and the rest of the procedure was as described above for Na^+,K^+-ATPase activity.

Measurement of *p*-nitrophenyl phosphate (*p*NPP) hydrolysis activity

Potassium-dependent *p*NPP hydrolysis activity, a partial reaction of Na^+,K^+-ATPase, was measured.[28] The concentrations of the various components in the 1 ml reaction mixture were 14–18 μg of enzyme, 25 mM sucrose, 0.1 mM EDTA, 100 mM Tris-HCl at pH 7.4, 15 mM KCl, 10 mM $MgCl_2$ and 24 mM *p*NPP. The reaction was started by the addition of *p*NPP at 37°C, and then stopped after 20 min with 1 ml of 5% Na_2CO_3 and 8% SDS. The color developed was read at 420 nm and the activity was estimated using 13.3 as the molecular extinction coefficient of *p*-nitrophenol.

Measurement of the amount of phosphointermediate

Formation of the phosphointermediate of Na^+,K^+-ATPase was measured at 0°C basically as described by Post and Sen.[29,30] Briefly, the enzyme was phosphorylated with [γ-^{32}P]ATP and detected with a liquid scintillation counter. The various components in the 50 μl reaction mixture were 10.4 μg of enzyme, 25 mM sucrose, 0.1 mM EDTA, 100 mM Tris-HCl (K^+ free) at pH 7.5, 5 mM NaCl, 1 mM $MgCl_2$ and 0.02 mM [γ-^{32}P]ATP. The reaction was started by the addition of ATP and terminated after 10 s with 4 ml of ice-cold 5% (w/v) trichloroacetic acid solution containing 3 mM ATP. The denatured phosphointermediate was trapped on the Whatman glass fiber filter and measured with a liquid scintillation counter.

Protein determination

The protein concentration was estimated by the method of Lowry *et al.*[31] with bovine plasma albumin as a standard.

Results

Concentration-dependent inhibition of Na^+,K^+-ATPase activity by cisplatin

Figure 1 shows the inhibition of Na^+,K^+-ATPase activity by cisplatin when the ATPase reaction was set to occur for 30 min in the presence of various concentrations of cisplatin. The activity decreased depending on the concentration of cisplatin, whereas more than 60% of control activity remained in the absence of cisplatin. As the inhibition curve seemed biphasic, we analyzed the concentration dependency in detail and found that the inhibition by cisplatin was also dependent on the time when the ATPase reaction with cisplatin was set off (data not shown).

Time-dependent inhibition of Na^+,K^+-ATPase by cisplatin

For further analysis of the time-dependent inhibition of Na^+,K^+-ATPase activity by cisplatin, the ATPase reaction was started after preincubation with cisplatin for periods of 0–180 min. As shown in Figure 2, the activity decreased depending on the preincubation time at each indicated concentration of cisplatin. We tried to determine the cisplatin concentration ($K_{i0.5}$) for half the maximal inhibition by plotting the remaining activity versus cisplatin concentration with the same preincubation time. The results showed that the $K_{i0.5}$ value decreased with longer preincubation time. For example, when the preincubation periods were 120 and 240 min, $K_{i0.5}$ values were about 250 and 130 μM, respectively (data not shown).

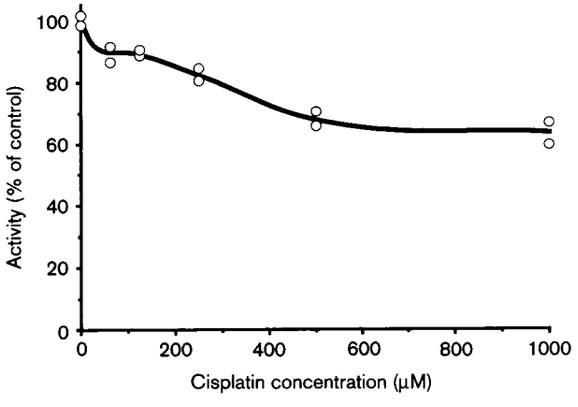

Figure 1. Concentration dependency of cisplatin effects on Na^+,K^+-ATPase activity. The enzyme was preincubated with 62.5, 125, 250, 500 or 1000 μM cisplatin added in the reaction mixture as described in Materials and methods for 30 min prior to start of the reaction. The reaction was started by adding 50 μl of ATP to give the final concentration of 5 mM to 250 μl of the reaction mixture at 37°C. After 20 min the reaction was stopped, and Na^+,K^+-ATPase activity was evaluated as described in Materials and methods. The data in the figure are shown as percentage values in which the Na^+,K^+-ATPase activity in the absence of cisplatin is shown as 100%. Duplicate experiments were done for each concentration of cisplatin.

Inhibition of Na^+-ATPase and K^+-dependent *p*NPPase activities by cisplatin

As our data showed that inhibition of Na^+,K^+-ATPase activity by cisplatin was both concentration and time dependent, we then examined which step in the reaction sequence of Na^+,K^+-ATPase was inhibited by cisplatin. In order to analyze the inhibition mechanism, we examined the effect of cisplatin on Na^+-ATPase and K^+-dependent *p*NPPase activities; these are partial reactions of Na^+,K^+-ATPase. The Na^+-ATPase activity is ATP hydrolysis activity observed without potassium. In this activity, phosphoenzyme spontaneously releases inorganic phosphate. K^+-dependent *p*NPPase activity is observed without sodium; this activity is believed to be caused by KE_2. As shown in Figures 3 and 4, both Na^+-ATPase and K^+-dependent *p*NPPase activities were inhibited by cisplatin; the degree of inhibition depended both on cisplatin concentration and preincubation time. However,

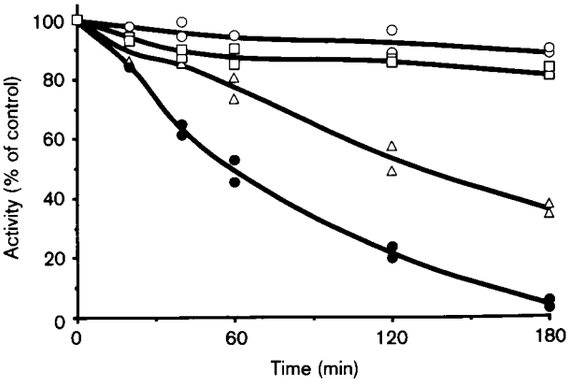

Figure 2. Preincubation time dependency of cisplatin effects on Na^+,K^+-ATPase activity. The enzyme was preincubated with 31.25 (○), 62.5 (□), 250 (△) or 500 (●) μM cisplatin added to the reaction mixture for various periods of time (0, 20, 40, 60, 120 and 180 min) at 37°C before determination of enzyme activity. The rest of the procedure was as described in the legend to Figure 1.

Na^+-ATPase was more sensitive to cisplatin than K^+-dependent *p*NPPase. When the preincubation time was 240 min, about 70% of Na^+-ATPase was inhibited by 500 μM cisplatin while inhibition of K^+-dependent *p*NPPase was less than 45%. As the results suggested that cisplatin more effectively inhibited the sodium-dependent step of the reaction sequence, we next tested the effects of cisplatin on the sodium-dependent formation of phosphointermediate.

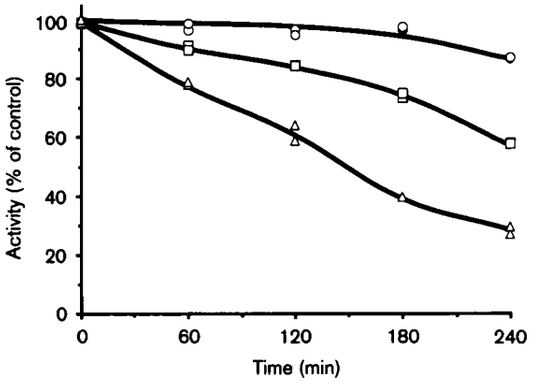

Figure 3. Effects of cisplatin on Na^+-ATPase activity. The enzyme was preincubated with 62.5 (○), 250 (□) or 500 μM (△) cisplatin in the reaction mixture during the period of time indicated at 37°C before determination of enzyme activities. Under the optimal condition for Na^+-ATPase activity described in Materials and methods, the reaction was started by adding 50 μl of ATP to give a final concentration of 2 mM to 250 μl of the reaction mixture without potassium at 37°C, and the mixture was incubated for 20 min. The rest of the procedure was as described in the legend to Figure 1.

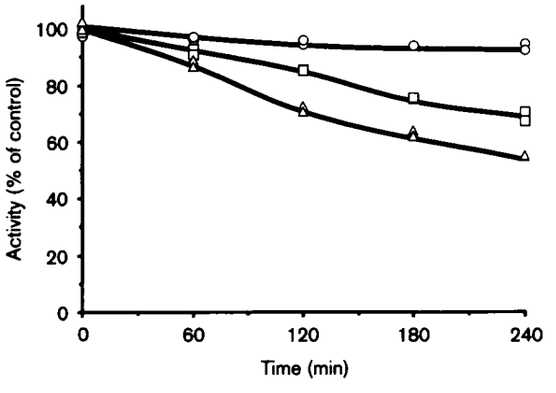

Figure 4. Effects of cisplatin on K^+-dependent pNPP hydrolysis activity. The enzyme was incubated with the same concentrations of cisplatin as in the measurement of Na^+-ATPase activity. Under the optimal conditions for K^+-dependent *p*NPP hydrolysis activity described in Materials and methods, the reaction was started by adding 200 μl of *p*NPP to give a final concentration of 24 mM to 800 μl of the reaction mixture without sodium at 37°C and the mixture was incubated for 20 min. The reaction was stopped and the enzyme activity was estimated as described in Materials and methods. The rest of the procedure was as described in the legend to Figure 1.

Inhibition of phosphointermediate formation by cisplatin

As shown in Figure 5, phosphointermediate (EP) formation after 180 min preincubation with various concentrations of cisplatin was inhibited to a degree depending on the cisplatin concentration; the inhibition was also dependent on the preincubation time (Figure 6). The decrease in the amount of EP formed after preincubation with 500 μM cisplatin correlated with the length of preincubation time. Thus the inhibition of EP formation by cisplatin was found to be dependent on both the preincubation time and the cisplatin concentration, as well as on the Na^+,K^+-ATPase, Na^+-ATPase and K^+-dependent *p*NPPase activity.

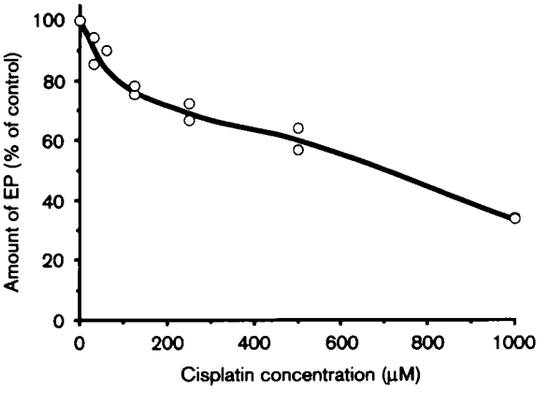

Figure 5. Concentration dependency of cisplatin effects on the amount of phosphointermediate. The specific activity of the enzyme was 1.2 μmol of P_i/mg protein/min at 37°C. The enzyme was preincubated with 31.25, 62.5, 125, 250, 500 or 1000 μM cisplatin for 120 min before measurement of phosphointermediate. The reaction was started by adding 5 μl of [γ-^{32}P]ATP to give a final concentration of 20 μM to 45 μl of the reaction mixture containing 10.4 μg of the enzyme and various concentrations of cisplatin indicated. After 10 s, the reaction was terminated by adding 4 ml of ice-cold solution containing 5% (w/v) trichloroacetic acid and 3 mM ATP. The amount of phosphointermediate was measured as described in Materials and methods. The amount of phosphointermediate in the presence of 40 mM KCl was subtracted as the background value. The rest of the procedure was as described in the legend to Figure 1.

Protection and recovery of cisplatin-inhibiting Na^+,K^+-ATPase activity by 2-mercaptoethanol (2-ME)

Since it is well known that sulfhydryls bind to and inactivate cisplatin,[32-34] we tested whether 2-ME, a sulfhydryl reagent, can protect Na^+,K^+-ATPase activity from the inhibitory effects of cisplatin. As shown in Figure 7, the addition of 4 or 8 mM 2-ME to cisplatin prevented the inhibition of Na^+,K^+-ATPase activity by cisplatin. Then we examined whether 2-ME could recover the activity already inhibited by cisplatin (Figure 8). At first, the enzyme was preincubated with 500 μM cisplatin for 120 min and about 20% of the activity was lost at this point. Then the solution to give 0, 4 or 8 mM of final 2-ME concentration was added to the cisplatin and preincubation was continued; Na^+,K^+-ATPase activity was measured at 180 and 240 min. Whereas the activity without 2-ME continued to decrease till 240 min, the one with 4 or 8 mM 2-ME was gradually recovered. On the other hand, when the cisplatin concentration was diluted with water from 250 to 22 μM at 120 min, the decrease in the activity was slower and the activity was not recovered (data not shown).

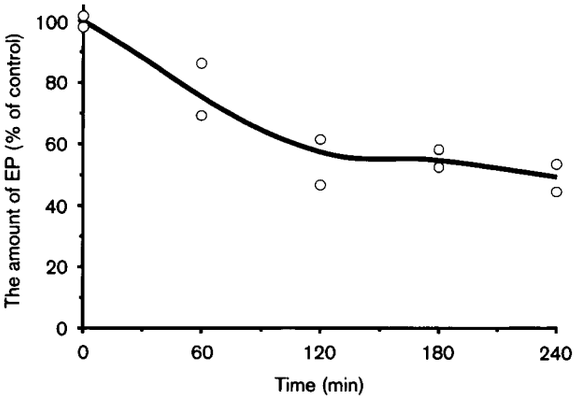

Figure 6. Preincubation time dependency of cisplatin effects on the amount of phosphointermediate. The same enzyme as used in Figure 5 was preincubated with 500 μM cisplatin for various periods indicated at 37°C before formation of the phosphointermediate. The rest of the procedure was as described in the legend to Figure 5.

Discussion

Cisplatin-concentration- and preincubation-time-dependent inhibition of Na^+,K^+-ATPase activity

It is possible that the inhibition of Na^+,K^+-ATPase activity by cisplatin is related to the accumulation of cisplatin itself in the cells,[12-16] nephrotoxicity[17-20] and the inhibition of methionine transport into cells.[8,9] Inhibition of methionine transport into cells may be the mechanism of biochemical modulation of cisplatin in the combination therapy with 5-FU.[5,6] Therefore it is important to understand the inhibition mechanism of Na^+,K^+-ATPase activity for optimal clinical use of cisplatin; however, much remains to be elucidated.

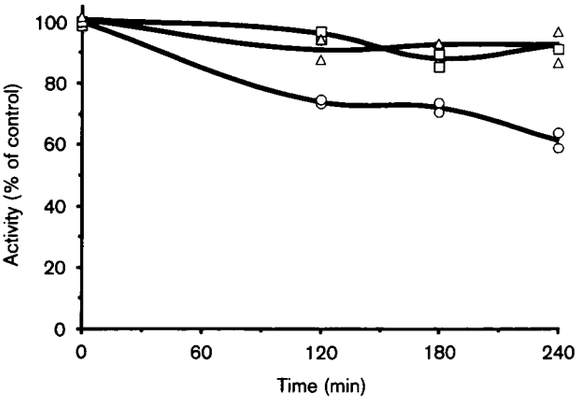

Figure 7. Protection of Na^+,K^+-ATPase activity by 2-ME from the inhibitory effect of cisplatin. The enzyme was preincubated with 500 μM cisplatin as described in the legend of Figure 2 without (○) or with 4 (□) or 8 (△) mM 2-ME during the various periods of time indicated and then enzyme activity was assayed. The rest of the procedure was as described in the legend to Figure 1.

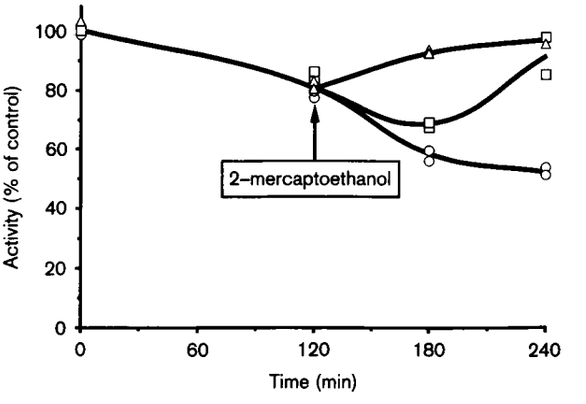

Figure 8. Recovery of Na^+,K^+-ATPase activity by 2-ME from inhibition by cisplatin. The enzyme was preincubated with 500 μM cisplatin as described in Figure 2 for 120 min and here solutions without (○) or with 4 (□) or 8 (△) mM 2-ME were added, respectively. The enzyme activity was assayed at the time indicated. The rest of the procedure was as described in the legend to Figure 1.

One of the problems in previous reports was that Na^+,K^+-ATPase was too crude for the study of the effect of cisplatin on the enzyme activity and that rubidium uptake was measured by the use of cells instead of measuring ATP hydrolysis activity directly.[12,14,15] These problems made it difficult to analyze the effect of cisplatin in detail. In fact, some of the previous results revealed that cisplatin-caused inhibition of Na^+,K^+-ATPase activity was both cisplatin-concentration- and preincubation-time-dependent, but they did not fully analyze the results.[19] We partially purified Na^+,K^+-ATPase from a human squamous cell carcinoma of the gingiva, Ca9-22 cells, which enabled us to study the inhibition mechanism in detail.

Our results clearly showed that the inhibition depended on both cisplatin concentration and time of preincubation with cisplatin (Figures 1 and 2). When the concentration of cisplatin was 250 μM, the activity slowly decreased until 420 min after start of the preincubation (data not shown). The concentration necessary to obtain half the maximal inhibition diminished when the preincubation time was longer. This suggests that it takes a long time before cisplatin exerts its biological effect and that the effect of cisplatin must be clinically evaluated long enough after administration. It is also important to arrest cell toxicity of cisplatin due to overdose. As a reason for the slow inhibition of Na^+,K^+-ATPase activity, Zwelling suggested that the active form of cisplatin, of which Cl residues are replaced with water, acquires reactivity with nucleophilic substances.[3] This change with water may need time and the slow inhibition of the enzyme activity in this study may reflect slow hydration of cisplatin. In clinical use as well, it would take time after administration for cisplatin to change into its active form.

Our results and those of others showed that cisplatin inhibits the Na^+,K^+-ATPase activity.[5,7,8,17–20] They also suggested that inhibition of Na^+,K^+-ATPase activity decreased the cellular accumulation of cisplatin.[12–15] These indicate that cisplatin administered as an anticancer reagent at the same time tends to decrease the accumulation of cisplatin in cancer cells through the inhibition of Na^+,K^+-ATPase activity. For this reason, cisplatin should perhaps be administered in as small as possible doses so as not to inhibit Na^+,K^+-ATPase activity and to be appropriately accumulated in the cells.

Inhibition mechanism of Na^+,K^+-ATPase activity by cisplatin

We compared the effects of cisplatin on Na^+-ATPase and K^+-dependent *p*NPPase activities, which are the partial reactions of Na^+,K^+-ATPase (Figures 3 and 4). The results showed that Na^+-ATPase was more sensitive to cisplatin than K^+-dependent *p*NPPase, suggesting that cisplatin more effectively inhibits the sodium-dependent step of the reaction sequence of Na^+,K^+-ATPase activity. Then we examined the effects of cisplatin on the sodium-dependent phosphointermediate formation (Figures 5 and 6), and found that cisplatin inhibited the formation in a concentration- and time-dependent manner, as observed in the inhibition of the activities. These results suggest that cisplatin mainly inhibits the activity in the step of ATP binding or phosphointermediate formation of Na^+,K^+-ATPase.

Recovery of Na^+,K^+-ATPase activity by 2-ME

Cisplatin is reported to be able to bind to thiol groups, and it has also been found that application of thiol compounds such as glutathione,[35,36] metallothionein[37,38] and diethyldithiocarbamate[32,34] reduces cisplatin-induced nephrotoxicity. These findings tempted us to test whether cisplatin-inhibiting Na^+,K^+-ATPase activity could be recovered by thiol compounds. As 2-ME, a thiol compound, has been used often for the study of Na^+,K^+-ATPase and is known to have an effect on the enzyme, we used 2-ME in this experiment. The presence of 4 or 8 mM 2-ME with 500 μM cisplatin protected the enzyme activity from the inhibitory effects of cisplatin almost completely (Figure 7). In a reverse experiment, we tried to recover the already inhibited activity by 500 μM cisplatin with 2-ME; as a result, the addition of 4 or 8 mM 2-ME to cisplatin recovered the enzyme activity almost completely (Figure 8). However, as the enzyme activity was not recovered by dilution with water (as already mentioned), the effect of 2-ME was responsible for the recovery of the activity. As Na^+,K^+-ATPase is known to have a cysteine residue near the active center, which is necessary for enzyme activity,[39,40] it is possible that cisplatin binds to this cysteine residue and inhibits the enzyme activity. In that case 2-ME may release cisplatin, bind to the residue itself and recover the activity of Na^+,K^+-ATPase. It also seemed possible that the affinity of cisplatin for 2-ME is stronger than that for the cysteine residue, and that 2-ME binds to cisplatin and releases it from the enzyme. However, we do not have direct evidence for this. Although the detailed mechanism is still unknown, the ability of 2-ME, a thiol compound, to recover the Na^+,K^+-ATPase activity which cisplatin has inhibited may be the reason for its ability to reduce nephrotoxicity.

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